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Characterization of Purinergic Receptors and Receptor-Channels Expressed in Anterior Pituitary Cells

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ABSTRACT

Purinergic G protein-coupled receptors (P2YR) and ion-conducting receptor-channels (P2XR) are present in the pituitary. However, their identification, expression within pituitary cell subpopulations, and the ability to elevate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to ATP stimulation were incompletely characterized. Here we show that mixed populations of rat anterior pituitary cells express messenger RNA transcripts for $P2Y_2R$, $P2X_{2a}R$, $P2X_{2b}R$, $P2X_3R$, $P2X_4R$, and $P2X_7R$. The transcripts and functional $P2Y_2R$ were identified in lactotrophs and GH3 cells, but not in somatotrophs and gonadotrophs, and their activation by ATP led to an extracellular Ca^{2+}-independent rise in $[Ca^{2+}]_i$ in about 40% of cells tested. Lactotrophs and GH3 cells, but not somatotrophs, also express transcripts for $P2X_7R$, $P2X_3R$, and $P2X_4R$. Functional $P2X_7R$ were identified in 74% of lactotrophs, whereas 50% of these cells expressed $P2X_3R$ and 33% expressed $P2X_4R$. Coexpression of these receptor subtypes in single lactotrophs was frequently observed. Purified somatotrophs expressed transcripts for $P2X_{2a}R$ and $P2X_{2b}R$, and functional receptors were identified in somatotrophs and gonadotrophs, but not in lactotrophs. Consistent with the cell-specific expression of transcripts for $P2X_2R$ and $P2X_3R$, the expression of their functional heteromers was not evident in pituitary cells. Receptors differed in their capacities to elevate and sustain Ca^{2+} influx-dependent rise in $[Ca^{2+}]_i$ during the prolonged ATP stimulation. These results indicate that the purinergic system of anterior pituitary is extremely complex and provides an effective mechanism for generating a cell- and receptor-specific Ca^{2+} signaling pattern in response to a common agonist. (*Endocrinology* **141:** 4091–4099, 2000)

PURINERGIC receptors are operative in a variety of tissues, including the pituitary gland (1). G protein-coupled adenosine receptors (ARs) and adenosine nucleotide-controlled receptors (P2YRs) and receptor-channels (P2XRs) are expressed in the pituitary. Adenosine 5′-triphosphate (ATP), the common and native agonist for all P2XRs and P2YRs, is secreted by anterior pituitary cells (2, 3) and the biological actions of ATP are terminated by ectonucleotidases (2). These enzymes degrade extracellular ATP in a sequential manner to adenosine, thereby activating ARs. The presence of phospholipase C-coupled P2YR was initially observed in a mixed population of sheep pituitary cells (4, 5). Single cell Ca^{2+} measurements have shown that activation of these receptors by ATP in rat pituitary cells is associated with an elevation in the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) derived from intracellular and extracellular sources (6–8). Molecular cloning and functional characterization of rat P2YRs in the pituitary revealed the presence of $P2Y_2R$ subtype with the pharmacological profile resembling the one observed in primary cultures of sheep pituitary cells (9). Pharmacological studies have also suggested the presence of P2XRs in pituitary cells (3, 10). The messenger RNAs (mRNAs) for $P2X_{2a}R$ and its spliced form, $P2X_{2b}R$, in pituitary cells have been identified recently (11, 12). Cultured and immortalized pituitary cells also express A_1-subtype of adenosine receptors, which are negatively coupled to adenylyl cyclase pathway. Receptor-mediated suppression of cAMP production together with the activation of inward rectifier potassium channels accounts for the inhibition of spontaneous electrical activity and PRL release (13, 14).

The presence of all three types of purinergic receptors in anterior pituitary is consistent with a view that ATP plays an important role in the control of Ca^{2+}-signaling and secretion in anterior pituitary. However, it has not been clarified as to which subtypes of P2 receptors are expressed and operative in the pituitary, nor the cell specificity in the expression/coexpression of these receptors. The focus in this study is on expression, distribution, and Ca^{2+} signaling function of P2YRs and P2XRs within three major subpopulations of secretory anterior pituitary cells, including lactotrophs, gonadotrophs, and somatotrophs. We screened the expression of all eight known P2XRs in cultured and immortalized pituitary cells. To help with the identification of receptor subtypes within these pituitary cells, we also expressed the P2XRs in GT1 hypothalamic neurons, which have a set of plasma membrane channels highly comparable to that of anterior pituitary cells (15, 16), but do not express P2YRs and P2XRs (12). The pharmacological and Ca^{2+}-signaling profiles of recombinant channels were compared with those observed in secretory anterior pituitary cells. The results of these investigations revealed an unusual complexity in the expression pattern and Ca^{2+}-signaling functions of these receptors and channels in the pituitary, especially in the lactotrophs.

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Materials and Methods

Cell cultures and transfection

Experiments were performed on anterior pituitary cells from adult female Sprague Dawley rats from Taconic Farms, Inc. (Germantown,

NY), as well as on immortalized GH3 pituitary cells and GT1 hypothalamic neurons. Pituitary cells were dispersed as previously described (12) and cultured in medium 199 containing Earle's salts, sodium bicarbonate, 10% horse serum, and antibiotics. GH3 and GT1 cells were cultured in DMEM medium containing 10% FCS (Life Technologies, Inc., Rockville, MD). Procedures for transient transfection in GT1 cells were performed as described (17) with minor modifications. Briefly, cells were plated on 35 mm coverslips coated with poly-L-lysine at a density of 7.5×10^4 cell per 35 mm dish and allowed to grow for 24 h. On the day of transfection, a total amount of 1.2 μg of expression constructs encoding P2XRs was mixed with 8 μl of cationic lipid Lipofectamine in a 1.2 ml of Opti-MEM medium (Life Technologies, Inc.), for 15 min at ambient temperature. The DNA mixture was then applied to cells for 3 h and replaced by normal culture medium. The cells were subjected to experiments 48 h after the transfection.

Cell purification

Separation of gonadotroph-, somatotroph-, and lactotroph-enriched populations was performed according to the size and density of pituitary cells, using the Eppendorf Cell Separation System. The one-liter disc-shaped chamber was filled from the bottom opening with about 900 ml of 2% to 4% Ficoll continuous gradient solution and then completed with 10% Ficoll solution. Dispersed cells (100×10^6) were suspended in 50 ml of 1% Ficoll solution and loaded into the chamber from the top opening. After adding 30 ml of separation medium on the top, the chamber was oriented to the horizontal position and was allowed to sediment at unit gravity for 2 h. The chamber was then reoriented to the inclined position and 15 fractions were collected from the bottom opening. Cells were washed, counted, and 10^5 cells from the unfractionated sample and from each separated fraction were suspended in 10 mM sodium carbonate, sonicated, and kept frozen at −20 C. Their hormonal contents were estimated by RIAs for rat LH, FSH, TSH, ACTH, PRL, and GH, which were done using the kits provided by Dr. Parlow and the National Hormone Pituitary Program. The highest concentrations of LH, TSH, GH, ACTH, and PRL were respectively found in fractions 1, 4, 8, 10, and 14. Estimated by the reverse hemolytic plaque assay, the percentage of purified cells was: 82% for LH-secreting cells, 68% for GH-secreting cells, and 85% for PRL-secreting cells. Purification of somatotrophs and lactotrophs was also done by a two-stage Percoll discontinuous density gradient centrifugation (18). Using the immunohistochemical staining approach, the purity of the enriched somatotroph and lactotroph fractions was estimated to be 92% and 64%, respectively.

Measurements of intracellular calcium ion concentration

For $[Ca^{2+}]_i$ measurements, cells were incubated in Krebs-Ringer buffer, supplemented with 2 μM fura-2 AM, at 37 C for 60 min. Coverslips with cells were washed with this buffer and mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attofluor Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD). Cells were examined under a 40× oil immersion objective during exposure to alternating 340 and 380 nm light beams, and the intensity of light emission at 520 nm was measured. The ratio of light intensities, F_{340}/F_{380}, which reflects changes in Ca^{2+} concentration, was followed in several single cells simultaneously at room temperature.

RT-PCR analysis of purinergic receptors and channels

Total RNA was isolated from mixed pituitary cells, or enriched somatotrophs and lactotrophs using TRIZOL reagent (Life Technologies, Inc.). First strand complementary DNA (cDNA) was then synthesized using a Superscript preamplification system (Life Technologies, Inc.). After RT using Superscript II RT and removal of RNA templates by RNase H digestion, a 5 μl-aliquot of first strand cDNA was used in subsequent PCR reactions. Primers for the P2XRs used in experiments are described in Ref (19). The $P2Y_2$ specific primers corresponded to the rat $P2Y_2R$ sequence: nucleotides 404 to 424 for the sense primer (5′-AACGGACGCTGAGCATCCAAG-3′) and 1611 to 1631 for the antisense primer (5′-TGAACTGACACCTGACTGAGC-3′). PCR conditions consisted of an initial denaturation step of 2 min at 94 C, followed by 20–25 cycles of denaturation at 94 C for 1 min, annealing at 60 C for 1 min, and extension at 72 C for 2 min. All PCRs were concluded with a final extension step of 10 min at 72 C. PCR samples were then size-fractionated in 1% agarose gel and visualized with ethidium bromide staining. To check for the integrity of RNA preparation, RT-PCR of GAPDH was also conducted as an internal control using primers GAPDH.S (5′-GGCATCCTGGGCTACACTG-3′) and GAPDH.AS (5′-TGAGGTCCACCACCCTGTT-3′) according to the PCR conditions reported previously (20). In these experiments, plasmids with the coding sequence of P2YR or P2XR cDNAs were used as the positive control for the respective PCRs.

Results

Distribution of P2YRs and P2XRs within the anterior pituitary cells

To characterize the distribution of P2YRs and P2XRs within the different cell types, ATP-induced $[Ca^{2+}]_i$ responses were analyzed in enriched subpopulations of secretory anterior pituitary cells. As discussed in *Materials and Methods*, purification of cells by Ficoll gradient procedure leads to 15 fractions of cells. Measurements of hormone contents in these fractions revealed that the first 3 fractions predominantly contained LH, fractions 3–5 TSH, fractions 5–10 GH, fractions 8–11 ACTH, and fractions 11–15 PRL. As shown in Fig. 1, the rise in $[Ca^{2+}]_i$ in response to GnRH was highly specific for gonadotrophs, as estimated by the parallelism between the profiles of GnRH responsive cells (B) and LH content in these cells (C), and by their inability to respond to TRH (E). On the other hand, there was a close correlation between PRL content profile (F) with that of TRH-responsive cells identified based on single cell Ca^{2+} measurements (E). The TSH-containing fractions 3–5 also responded to TRH (E). A small fraction of unidentified cells (1–2%) from fraction 9 responded to both TRH and GnRH (see also Ref. 21). Thus, these isolation procedures provide an effective system to enrich gonadotrophs and lactotrophs, to separate the TRH-sensitive thyrotrophs and lactotrophs, and to identify single gonadotrophs and lactotrophs with respect to GnRH- and TRH-induced $[Ca^{2+}]_i$ responses.

In Ca^{2+}-containing medium, 100 μM ATP increased $[Ca^{2+}]_i$ in 50–90% of the cells in different fractions, suggesting that purinergic receptors are native to all subpopulations of cells (Fig. 1A). The majority of GnRH-responsive cells also responded to ATP when bathed in Ca^{2+}-containing medium (B), and only 1–2% of these cells responded to ATP when bathed in Ca^{2+}-deficient medium (D). In lactotroph fractions of pituitary cells, however, ATP elevated $[Ca^{2+}]_i$ in about 40% of cells in Ca^{2+}-deficient medium (D) and in 80–90% of cells in Ca^{2+}-containing medium (A). A group of unidentified cells elutriated with somatotroph/corticotroph fractions also responded to ATP with an extracellular Ca^{2+}-independent rise in $[Ca^{2+}]_i$ (fractions 5–10). These results indicate that Ca^{2+}-mobilizing P2YRs are predominantly expressed in lactotrophs, whereas all subpopulations of anterior pituitary cells expressed P2XRs capable of facilitating Ca^{2+} influx.

Characterization of pituitary P2YRs

The pharmacological profile of P2YRs expressed in lactotrophs is shown in Fig. 2. In addition to ATP, these receptors also responded to uridine triphosphate (UTP), a specific agonist for $P2Y_2R$ and $P2Y_4R$, and adenosine-5′-O-(3-thiotriphosphate) (ATP-γ-S) when bathed in Ca^{2+}-deficient me-

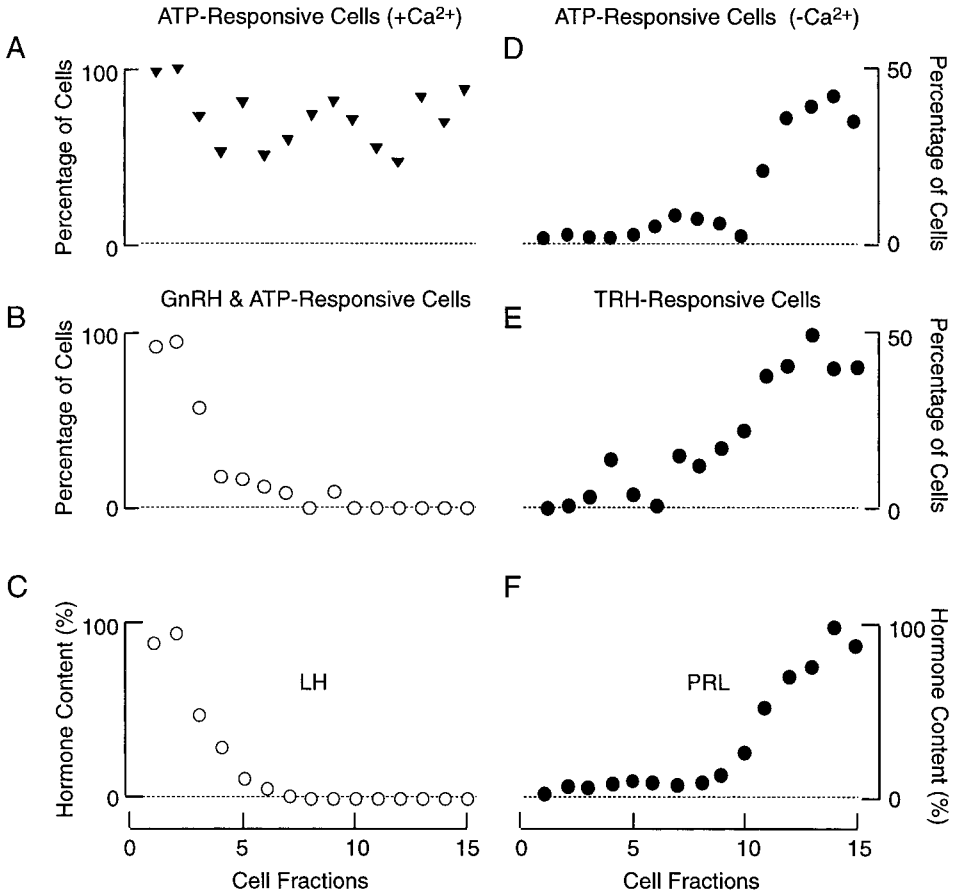

FIG. 1. Distribution of purinergic receptors and receptor-channels within the subpopulations of anterior pituitary cells. Dispersed cells were separated by Ficoll gradient into 15 fractions as described in *Materials and Methods*. A and B, Percentage of cells responding to ATP (A) and ATP + GnRH (B), both when bathed in Ca^{2+}-containing medium. C, LH content in different fractions (normalized values). D and E, Percentage of cells responding to ATP (D) and TRH (E) when bathed in Ca^{2+}-deficient medium. F, PRL content in different fractions (normalized values). In A, B, D, and E, number of cells analyzed for $[Ca^{2+}]_i$ response per fraction varied between 25 and 62. In C and F, 10^5 cells were dialyzed immediately after separation to measure LH and PRL cell content.

dium (Fig. 2A). The relative potency of ATP agonists for these receptors was ATP > UTP > ATP-γ-S. We have also examined the effects of 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP), a relatively specific agonist for $P2X_7R$ (22), on $[Ca^{2+}]_i$ response in lactotrophs bathed in Ca^{2+}-deficient medium. As shown in Fig. 2A, BzATP was unable to initiate Ca^{2+} signaling in lactotrophs. Several other ATP analogs, including α,β-methylene ATP (α,β-meATP; Fig. 2A) and 2-methylthio-ATP (2-MeS-ATP; not shown) were also ineffective in generating Ca^{2+} signals in lactotrophs bathed in Ca^{2+}-deficient medium. Such a pharmacological profile of $[Ca^{2+}]_i$ responses was typical for all responding cells, suggesting that these cells express $P2Y_2R$ subtype. In accordance with the results of pharmacological characterization, RT-PCR using $P2Y_2R$ primers also detected a PCR fragment with the expected size of $P2Y_2R$ in enriched lactotrophs. This specific message was also identified in immortalized GH3 cells (Fig. 2B).

Expression of P2XR transcripts in anterior pituitary cells

Molecular identification of P2XRs expressed in anterior pituitary cells was also obtained by RT-PCR analysis. Using specific rat $P2X_2R$ primers, which covered the entire open reading frame of P2XR cDNA sequence, two sizes of PCR products of about 1.6 kb and 1.4 kb long were detected in mixed populations of pituitary cells (Fig. 3A, lane 1). As described in (12), these products corresponded to $P2X_{2a}R$ and its spliced form, $P2X_{2b}R$, and their presence in the mixed population of pituitary cells was confirmed by specific primers. The mRNAs for these channels were observed in somatotrophs purified by two-stage Percoll discontinuous density gradient centrifugation (Fig. 3C, lane 2).

In addition to $P2X_2R$ transcripts, mixed anterior pituitary cells also expressed transcripts for $P2X_3R$, $P2X_4R$, and $P2X_7R$ (Fig. 3A, lane 1). Immortalized GH3 pituitary cells also expressed transcripts for $P2X_3R$, $P2X_4R$, and $P2X_7R$, but not $P2X_{2a}R$ and $P2X_{2b}R$ (Fig. 3A, lane 4). In parallel to GH3 transcript profile, the messages for $P2X_3R$, $P2X_4R$, and $P2X_7R$ were found in purified lactotrophs (Fig. 3B, lane 4), but not in somatotrophs (lane 2). Finally, the transcripts for $P2X_1R$, $P2X_5R$, and $P2X_6R$ were not observed in mixed pituitary cells (Fig. 3A, lane 1) and GH3 cells (Fig. 3A, lane 4).

Calcium signaling by recombinant channels

To help identify functional P2XR subtypes present in anterior pituitary cells, the recombinant channels were expressed in GT1 neurons and their pharmacological and $[Ca^{2+}]_i$ signaling profiles were compared with those observed in lactotrophs, somatotrophs, and gonadotrophs. As shown in Fig. 4, GT1 cells expressing homomeric $P2X_3R$ responded to αβ-methylene ATP (αβ-meATP) with a small amplitude and transient rise in $[Ca^{2+}]_i$. In accord with the literature data (19, 23, 24), the heteromeric $P2X_2R$ + $P2X_3R$ channels also responded to this agonist (Fig. 4A). However, the amplitude of αβ-meATP-induced $[Ca^{2+}]_i$ responses in cells expressing $P2X_2R$ + $P2X_3R$ was higher compared with

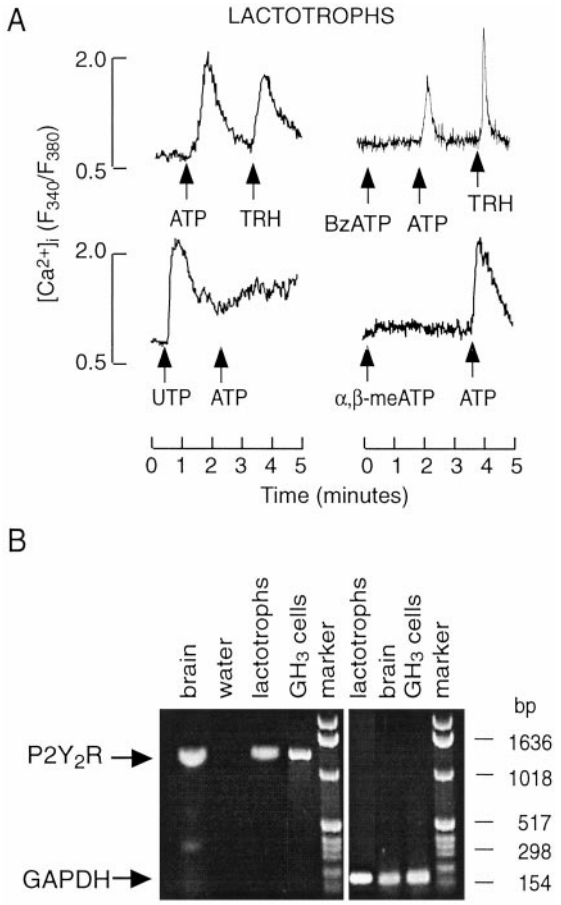

FIG. 2. Characterization of P2YR expressed in lactotrophs. A, Pattern of $[Ca^{2+}]_i$ response in purified lactotrophs. *Arrows* indicate the moment of agonist application. The final concentration of ATP and its analogs was 100 μM. At the end of each experiment, 100 nM TRH was added. Experiments were performed in Ca^{2+}-deficient medium. About 40% of lactotrophs responded to ATP in such conditions. B, RT-PCR analysis of P2YR mRNA expression in enriched lactotrophs and GH3 cells (*left panel*). PCR was conducted using primers specific for rat $P2Y_2R$. PCR products were then separated in 1% agarose gel and visualized with ethidium bromide. RT-PCR of glyceraldehyde phosphate dehydrogenase (GAPDH, *right panel*) was performed to monitor the quality of RNA preparation. Total RNA isolated from the rat brain was used as the positive control. In the case of "no template" control, water was substituted for the first strand cDNA sample in PCR reaction.

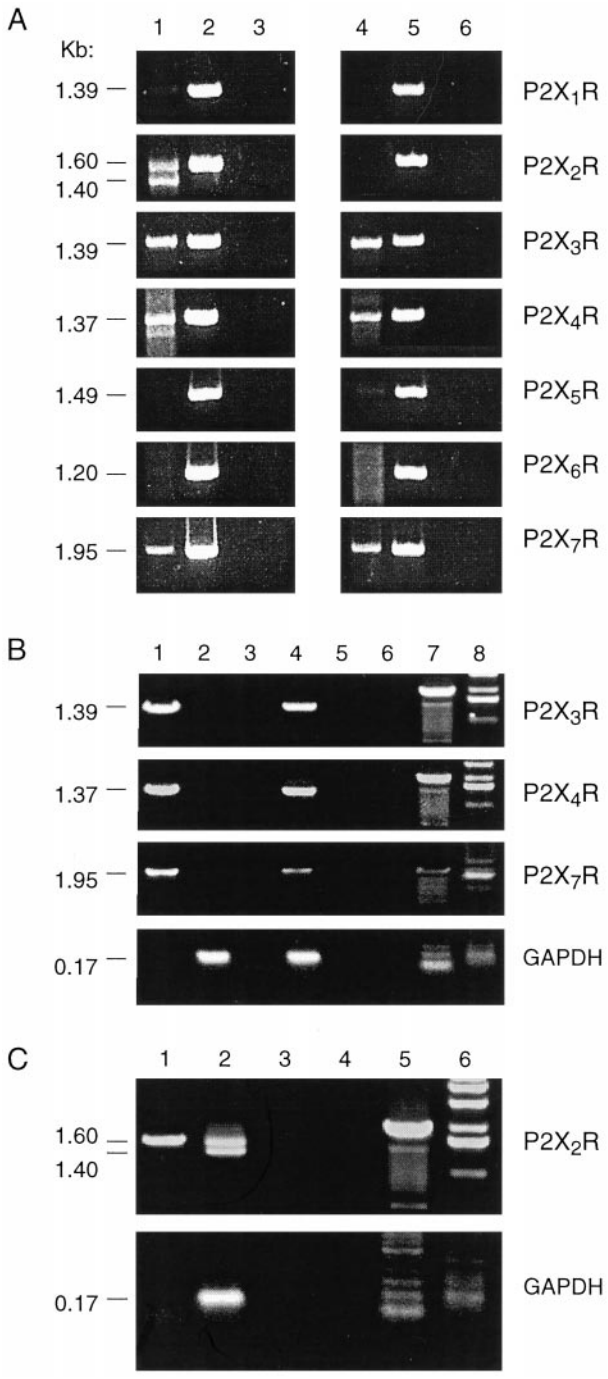

FIG. 3. Expression of P2XRs in pituitary cells. A, Detection of P2XRs in mixed population of anterior pituitary cells (lane 1) and immortalized GH3 cells (lane 4). B, Expression of $P2X_3R$, $P2X_4R$, and $P2X_7R$ mRNA in enriched somatotrophs (lane 2) and lactotrophs (lane 4). C, Expression of $P2X_2R$ mRNA in enriched somatotrophs (lane 2). Plasmids containing the coding sequence of the respective P2XR cDNAs were used as positive controls (A, lanes 2 and 5; B and C, lane 1). For negative controls, PCR was conducted using first strand cDNA samples without RT (A, lanes 3 and 6; B, lanes 3 and 5; C, lane 3). In the case of "no template" control, water was substituted for first strand cDNA sample in PCR reactions (B, lane 6; C, lane 4). DNA markers are shown in lanes 7 and 8 (B) and 5 and 6 (C). Primer sequences are described in Ref. 19. A 10 μl-aliquot of PCR products was analyzed in 1% agarose gel containing ethidium bromide. GAPDH primers were used as an internal control to monitor the quality of RNA preparation.

that observed in $P2X_3R$-expressing cells (Fig. 4A *vs.* 4B). Cells expressing $P2X_{2a}R$, $P2X_{2b}R$, $P2X_4R$, and $P2X_7R$ were insensitive to $\alpha\beta$-meATP (Fig. 4, C–F). These results indicate that $\alpha\beta$-meATP can be used in $[Ca^{2+}]_i$ measurements, as in current measurements (23) as a highly selective agonist for the identification of functional $P2X_3R$ homomers and $P2X_2R$ + $P2X_3R$ heteromers.

Another agonist, 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP) was able to elevate $[Ca^{2+}]_i$ in cells expressing $P2X_7R$, $P2X_{2a}R$, and $P2X_{2b}R$, whereas $P2X_3R$- and $P2X_4R$-expressing cells were practically insensitive to this agonist (Fig. 5A). In $P2X_7R$-expressing cells, addition of 100 μM ATP in the presence of 100 μM BzATP was ineffective (Fig. 5A, *bottom trace*), whereas in all $P2X_{2a}R$ and $P2X_{2b}R$-expressing cells ATP was able to further elevate $[Ca^{2+}]_i$ (two top traces). This suggests

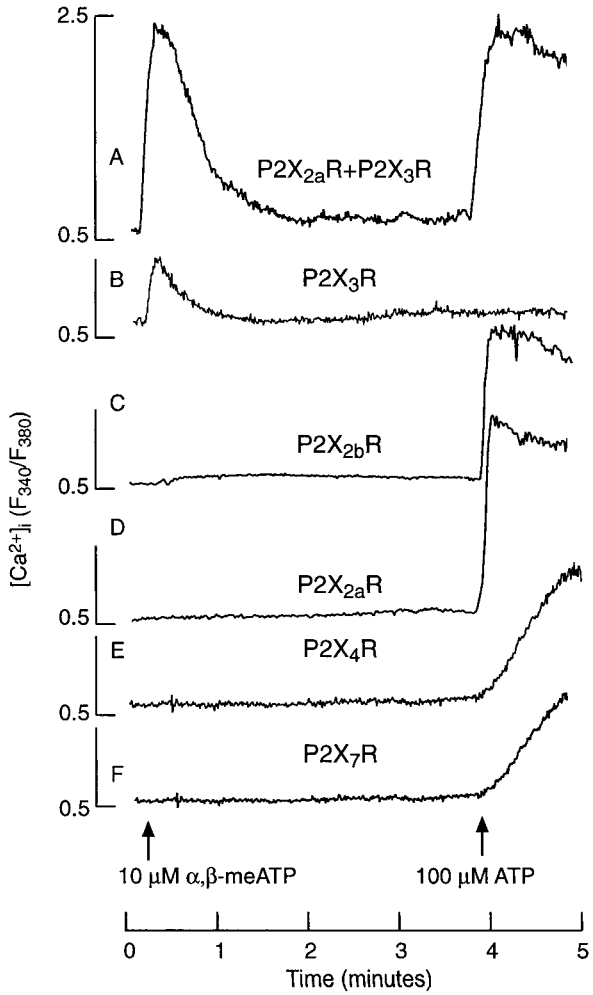

FIG. 4. Effects of α, β-meATP, the specific agonist for $P2X_3R$, on Ca^{2+} influx in GT1 neurons transiently expressing the recombinant P2XRs. Cells were transfected with one of the P2XRs, or in combination (*top trace*) and were subjected to stimulation 48 h after the transfection. ATP was added in the presence of α, β-meATP. The traces shown are representative from at least 20 records for each receptor.

that $P2X_7R$ is more sensitive to BzATP, contrary to $P2X_{2a}R$ and $P2X_{2b}R$, which are more sensitive to ATP. Consistent with this, BzATP induced an additional rise in $[Ca^{2+}]_i$ in $P2X_7R$-expressing cells already stimulated with ATP, 2-MeS-ATP, or ATP-γ-S (Fig. 5B, *right traces*) but was ineffective in $P2X_{2a}R$-expressing cells stimulated with these agonists (Fig. 5B, *left traces*). Such pharmacological profiles of $P2X_2R$ and $P2X_7R$ provide an effective tool to identify cells expressing these two channels.

The BzATP-insensitivity of $P2X_4R$ (Fig. 5A) is a useful but not sufficient tool in a search for cells expressing these channels. However, only two cloned channels, $P2X_4R$ and $P2X_6R$, are insensitive to suramin, a P2XR antagonist (25). As shown in Fig. 6A, ATP-induced $[Ca^{2+}]_i$ response in $P2X_4R$-expressing cells was not affected by the addition of 200 μM suramin for 3 min before the application of ATP. Because the message for $P2X_6R$ was not observed in pituitary cells (Fig. 3), the insensitivity of $P2X_4R$ channels to suramin was employed in a search for their expression in pituitary cells.

Characterization of P2XR expressed in pituitary cells

In a mixed population of anterior pituitary cells, $\alpha\beta$-meATP, the specific agonist for $P2X_3R$ and $P2X_2R$ + $P2X_3R$, was able to induce rise in $[Ca^{2+}]_i$ only in TRH-sensitive cells. A fraction of lactotrophs, but not gonadotrophs and somatotrophs, also responded to $\alpha\beta$-meATP with a rise in $[Ca^{2+}]_i$, the pattern of which was highly comparable to that observed in GT1 neurons expressing homomeric $P2X_3R$ (Fig. 7A *vs.* 4B). None of the $\alpha\beta$-meATP-sensitive cells generated $[Ca^{2+}]_i$ signals with the amplitude and duration comparable to that observed in cells bearing heteromeric $P2X_2R$ + $P2X_3R$. This is consistent with the absence of $P2X_2R$ transcripts in immortalized GH3 cells (Fig. 3A) and $P2X_3R$ transcripts in purified somatotrophs (Fig. 3B), further suggesting that lactotrophs only express $P2X_3R$ homomers. Table 1 illustrates that about 50% of lactotrophs express these channels.

On the other hand, BzATP, an agonist that activates $P2X_7R$ and $P2X_2R$, induced rises in $[Ca^{2+}]_i$ in a majority of lactotrophs, somatotrophs, and gonadotrophs. About 75% of lactotrophs (Table 1) responded to BzATP with the nondesensitizing $[Ca^{2+}]_i$ response, typically observed in $P2X_7R$-expressing cells. Consistent with this conclusion, addition of ATP, ATP-γ-S, and 2-MeS-ATP in the presence of BzATP was ineffective in all lactotrophs studied. Figure 7B illustrates three such cells. In contrast to lactotrophs, addition of ATP, ATP-γ-S, and 2-MeS-ATP in the presence of BzATP, induced further increase in $[Ca^{2+}]_i$ in somatotrophs and gonadotrophs (Fig. 8), indicating that these cells express $P2X_2R$. In accordance with this, BzATP was unable to elevate $[Ca^{2+}]_i$ in the presence of ATP, ATP-γ-S, or 2-MeS-ATP (Fig. 8, *right tracings*). This pattern of response was observed in 84% of gonadotrophs, and 82% of somatotrophs (Table 1).

Finally, in a fraction of lactotrophs (Table 1), 100 μM ATP induced suramin-insensitive $[Ca^{2+}]_i$ response, the pattern of which was highly comparable to that observed in GT1 neurons expressing homomeric $P2X_4R$. Figure 6B illustrates a cell responding to ATP in suramin-containing and -deficient medium. Such a pattern of $[Ca^{2+}]_i$ response was observed in about 33% of lactotrophs (Table 1). These results indicate that somatotrophs and gonadotrophs exclusively express $P2X_2R$, whereas lactotrophs express $P2X_3R$, $P2X_4R$, and $P2X_7R$, in addition to already characterized $P2Y_2R$ (6).

The coexpression of P2XRs in single lactotrophs was frequently observed. A fraction of cells expressing the α,β-meATP-sensitive $P2X_3R$, also responded to the application of BzATP and ATP with additional rise in $[Ca^{2+}]_i$, presumably by activating $P2X_7R$ (Fig. 7A, *two bottom traces*). Similarly, about 15% of lactotrophs responded to BzATP with a nondesensitizing response and ATP with a spike response, suggesting the expression of both $P2X_7R$ and $P2Y_2R$ in the same cells (Fig. 7C, *upper trace*). Consistent with this, removal of extracellular Ca^{2+} abolished BzATP-induced rise in $[Ca^{2+}]_i$ and the subsequent addition of ATP in Ca^{2+}-deficient medium generated a typical Ca^{2+}-mobilizing spike response. Finally, ATP was able to induce a suramin-insensitive rise in $[Ca^{2+}]_i$ in a small fraction of lactotrophs already stimulated with BzATP, the profile of which was comparable to that observed in GT1 neurons expressing $P2X_4R$ (not shown).

In addition to the variable agonist sensitivity, pituitary P2XRs also differed with respect to their peak amplitude $[Ca^{2+}]_i$ responses and temporal response patterns. As shown

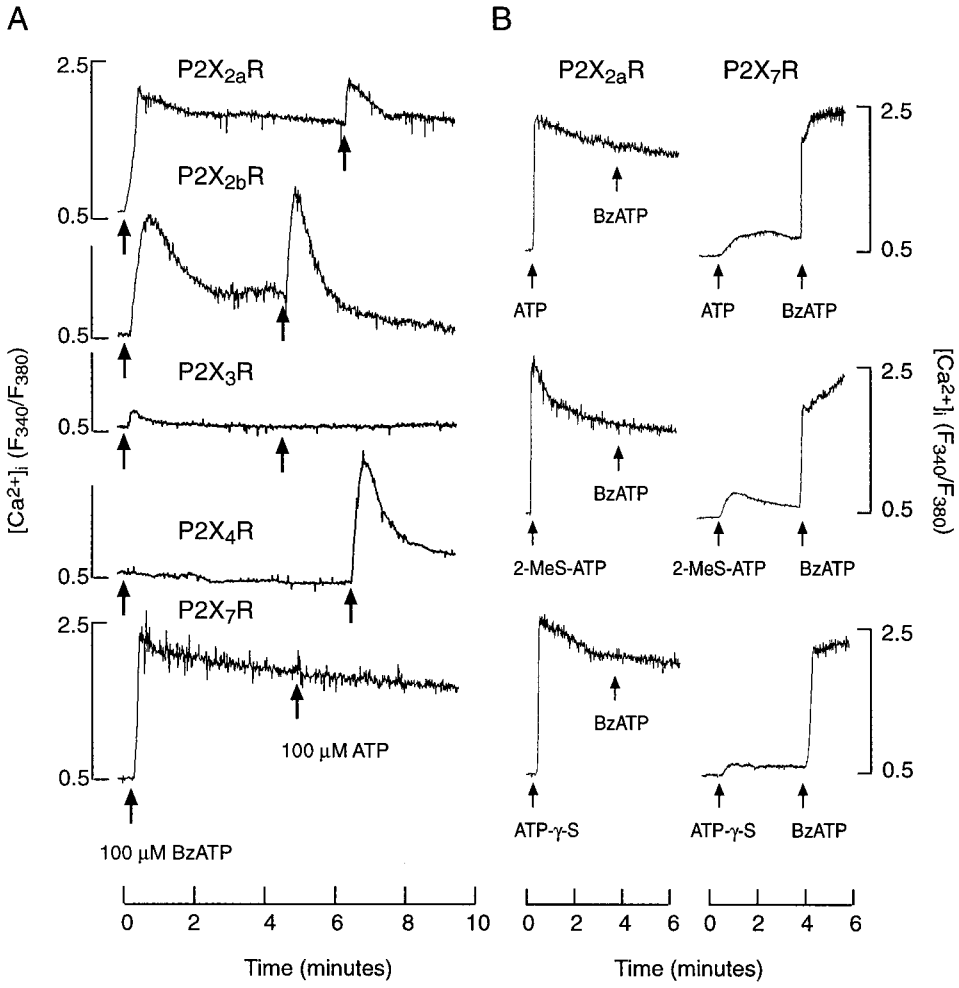

FIG. 5. Effects of BzATP on Ca^{2+} influx in GT1 neurons transiently expressing the recombinant P2XRs. A, Effects of BzATP and ATP on $[Ca^{2+}]_i$ in cells expressing different P2XRs. ATP was added in the presence of BzATP. B, Comparison of the effects of BzATP *vs.* other ATP analogs on $[Ca^{2+}]_i$ in cells expressing $P2X_{2a}R$ (*left traces*) and $P2X_7R$ (*right traces*).

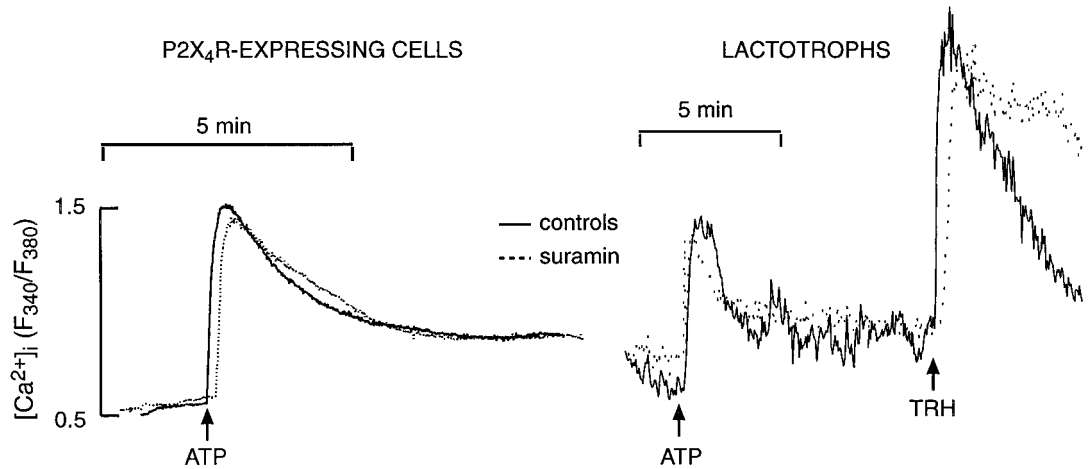

FIG. 6. Characterization of $P2X_4R$ in GT1 neurons and lactotrophs. A, Suramin-insensitivity of recombinant $P2X_4R$ expressed in GT1 neurons. The tracings shown are averaged data from 20 records. B, Suramin-insensitivity of ATP-induced $[Ca^{2+}]_i$ response in a fraction of lactotrophs.

in Table 2, the highest amplitude of $[Ca^{2+}]_i$ response was observed in pituitary gonadotrophs and somatotrophs expressing $P2X_2R$. The peak amplitudes generated by $P2X_7R$ in lactotrophs were somewhat smaller, whereas the peak amplitude of $[Ca^{2+}]_i$ response in lactotrophs expressing $P2X_3R$ and $P2X_4R$ was about 40% of that observed in cells expressing $P2X_2R$. The native receptors also desensitized with different rates: $P2X_3R > P2X_4R > P2X_2R > P2X_7R$ (Table 2).

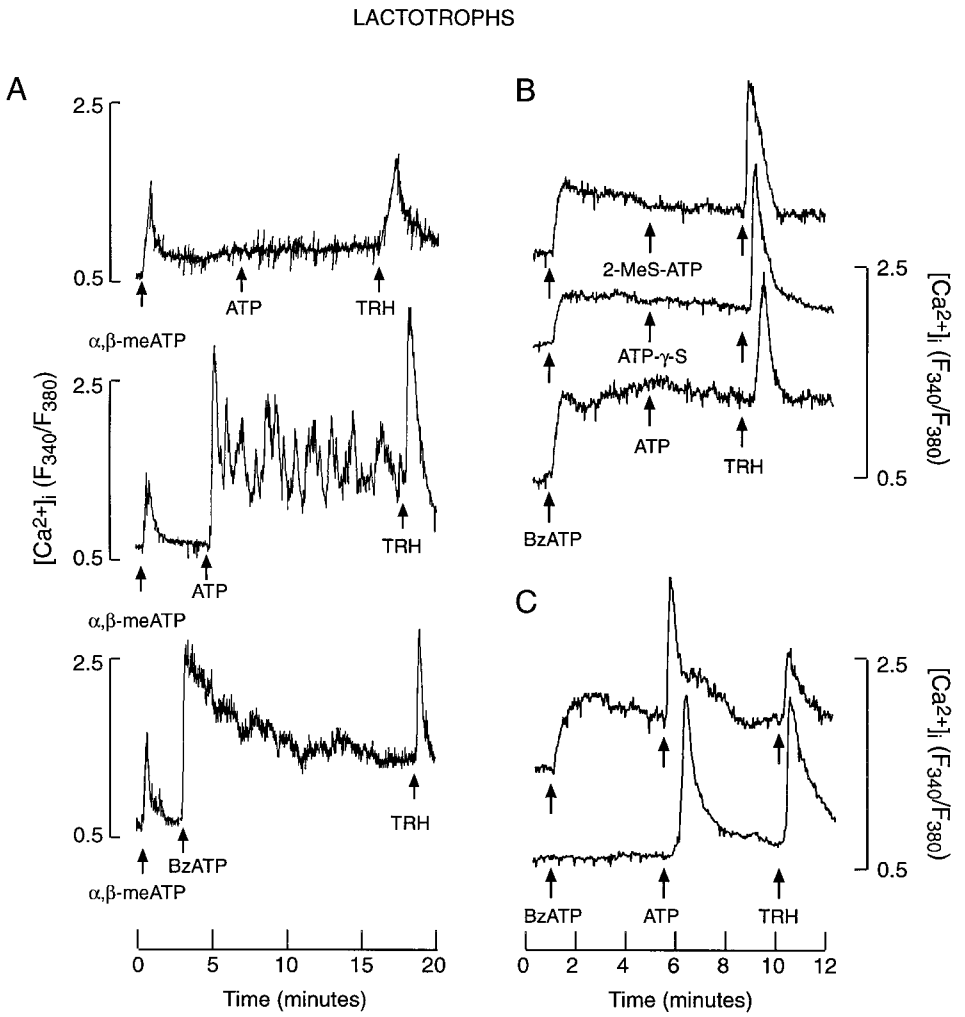

FIG. 7. Pharmacological characterization of P2XR and P2YR in lactotrophs. A, Characterization of α, β-meATP-sensitive $P2X_3R$ in lactotrophs. *Top trace*, Lactotroph responding to α, β-meATP, the specific agonist for $P2X_3R$, but not to ATP. *Bottom traces*, Cells responding to both α, β-meATP and ATP or BzATP, which were added in the presence of α, β-meATP. B, Characterization of $P2X_7R$ expressed in lactotrophs by comparing the potency of ATP and its analogs in promoting Ca^{2+} influx. C, Coexpression of BzATP-sensitive $P2X_7R$ and BzATP-insensitive $P2Y_2R$ in lactotrophs.

TABLE 1. Distribution of functional P2XRs in anterior pituitary cells

Cell type:	$P2X_2R$	$P2X_3R$	$P2X_4R$	$P2X_7R$
Gonadotrophs	49/58 (84%)	0/57 (0%)	—	0/58 (0%)
Somatotrophs	46/56 (82%)	0/42 (0%)	—	0/56 (0%)
Lactotrophs	0/37 (0%)	34/68 (50%)	14/42 (33%)	52/70 (74%)

$P2X_2Rs$ were identified by an additional $[Ca^{2+}]_i$ response to 100 μM ATP in cells already stimulated with 100 μM BzATP, whereas the nonresponders were defined as $P2X_7R$-expressing cells. $P2X_3Rs$ were identified by their $[Ca^{2+}]_i$ responses to α,β-meATP. Suramin insensitivity was employed in a search for $P2X_4R$ in lactotrophs. For detail on criteria and experimental strategy for identifying P2XR in pituitary cells see the section on *Calcium signaling by recombinant channels*.

Discussion

Functional purinergic receptors and receptor-channels are present in all subpopulations of pituitary cells, indicating that ATP acts as a nonselective agonist in the control of pituitary functions (10). However, the identification of receptor subtypes expressed in the pituitary and the selectivity of their expression in individual secretory cell types were incompletely characterized. Here we have identified the P2XR and P2YR subtypes that are operative in anterior pituitary and have characterized their expression in three secretory cell types: gonadotrophs, somatotrophs, and lactotrophs. Our RT-PCR results indicate that mixed pituitary cells express mRNA transcripts for $P2Y_2R$, $P2X_{2a}R$, and $P2X_{2b}R$. In addition to those transcripts that have been previously found in mixed pituitary cells (9, 12), we have also identified transcripts for $P2X_3R$, $P2X_4R$, and $P2X_7R$. The transcripts for $P2Y_2R$, $P2X_3R$, $P2X_4R$, and $P2X_7R$ were found in lactotrophs, but not in somatotrophs, which exclusively express transcripts for $P2X_{2a}R$ and $P2X_{2b}R$. The RT-PCR analysis of P2XRs in gonadotrophs was inconclusive, probably because of the low number of cells obtained by our cell purification procedures. Consistent with the results of RT-PCR analysis, single cell $[Ca^{2+}]_i$ measurements indicate that lactotrophs express functional $P2Y_2R$, as well as $P2X_3R$, $P2X_4R$, and $P2X_7R$, whereas somatotrophs express $P2X_2R$. The extensive pharmacological characterization of P2XRs in gonadotrophs also indicates that these cells express exclusively $P2X_2R$ subtypes.

When activated, these receptor-channels generate $[Ca^{2+}]_i$ signals of different amplitude and duration. $P2X_3R$ generates small amplitude $[Ca^{2+}]_i$ signals, which desensitize within 1–2 min. In contrast, $P2X_7R$ generates high amplitude $[Ca^{2+}]_i$ signals that do not desensitize during the prolonged agonist stimulation. $P2X_2Rs$ expressed in gonadotrophs and soma-

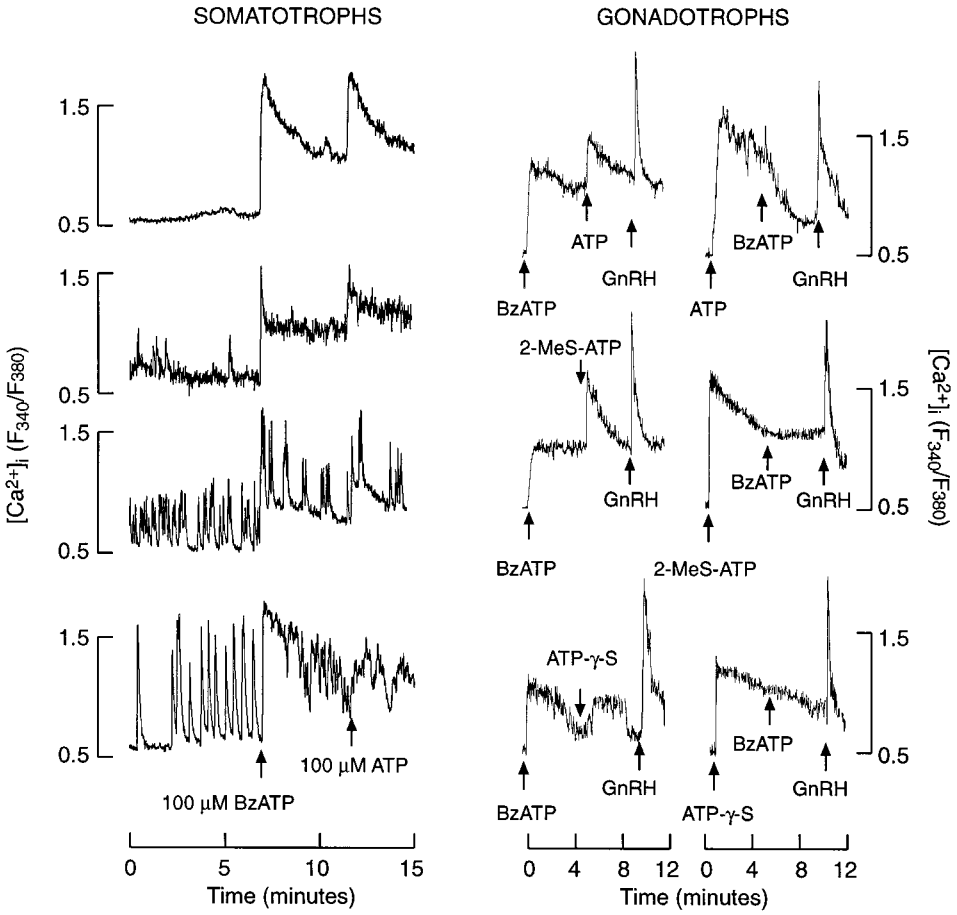

FIG. 8. Pharmacological characterization of $P2X_2R$ expressed in somatotrophs (*left traces*) and gonadotrophs (*right traces*). In somatotrophs, the traces shown are typical of those observed in quiescent cells (*top trace*) and spontaneously active cells (*three bottom tracings*). *Arrows* at bottom illustrate the moment of drug application in all traces shown. In gonadotrophs, the majority (about 80%) of cells were quiescent, and tracings shown are representative from such cells.

TABLE 2. Characterization of agonist-induced $[Ca^{2+}]_i$ responses in pituitary cells

Channels-Cells	Agonist (100 μM)	Peak $[Ca^{2+}]_i$ (F_{340}/F_{380})	Desensitization rate (sec^{-1})
$P2X_2R$—somatotrophs	ATP	1.274 ± 0.058 (45)	0.010 ± 0.008 (12)
$P2X_2R$—gonadotrophs	ATP	1.140 ± 0.062 (14)	0.011 ± 0.007 (13)
$P2X_3R$—lactotrophs	α,β-me-ATP	0.422 ± 0.022 (34)	0.037 ± 0.005 (9)
$P2X_4R$—lactotrophs	ATP[a]	0.459 ± 0.043 (14)	0.019 ± 0.008 (5)
$P2X_7R$—lactotrophs	BzATP	0.980 ± 0.050 (22)	Non-desensitizing

The peak $[Ca^{2+}]_i$ responses are expressed as the difference in the basal and peak $[Ca^{2+}]_i$ responses, shown as F_{340}/F_{380}.

[a] Experiments were done in the presence of 100 μM suramin. The time course of $[Ca^{2+}]_i$ response to ATP was fitted to an exponential function using GraphPad Prism (GraphPad Software). The values shown are means ± SEM.

totrophs are able to generate high amplitude $[Ca^{2+}]_i$ signals, but they desensitize gradually and lead to the attenuation of Ca^{2+} signals to about 10–20% of that observed at the beginning of stimulation. In general, such pattern of Ca^{2+} signals resembles those observed in GT1 neurons transiently expressing recombinant P2XRs. However, in the case of $P2X_7R$, there are differences between the peak amplitude of $[Ca^{2+}]_i$ responses in pituitary cells *vs.* that of GT1 cells when stimulated with BzATP, which may be result of overexpression of these channels in host cells. The recombinant $P2X_{2a}R$ also desensitizes incompletely in GT1 cells, but the time needed to reach the steady-state signaling is prolonged when compared with the native $P2X_2R$s expressed in pituitary cells. This suggests that the mechanism of controlling Ca^{2+} influx through $P2X_2R$ is different between pituitary cells and GT1 cells used in our expression studies.

The coexpression of $P2X_2R$ with $P2X_3R$ provides an effective mechanism to control Ca^{2+} influx through $P2X_{2a}R$ (23, 24). Although the transcripts for $P2X_3R$ are present in pituitary cells; however, several lines of evidence argue against the existence of $P2X_{2a}R + P2X_3R$ heteromers in pituitary cells. Purified lactotrophs and immortalized GH3 cells expressed the transcripts for $P2X_3R$, whereas the transcripts for $P2X_{2a}R$ and $P2X_{2b}R$ were identified in somatotrophs. In parallel to that, functional $P2X_3R$ was identified in lactotrophs, as well as in an unidentified cell type, but not in gonadotrophs and somatotrophs. Finally, none of the α,β-meATP-sensitive cells from mixed subpopulations exhibited the pattern of Ca^{2+} signals generated by the recombinant $P2X_{2a}R + P2X_3R$ heteromers. The cell specific expression of $P2X_2R$ and $P2X_3R$ in anterior pituitary probably accounts for the lack of coexpression of such heteromers.

On the other hand, we (12) and others (26, 27) reported recently that the control of Ca^{2+} influx through $P2X_2R$ can be achieved by the coexpression of $P2X_{2a}R$ and $P2X_{2b}R$. Depending on the assemble mode, such heteromers can desen-

sitize with variable rates (12). The Ca^{2+}-signaling profiles in pituitary cells expressing $P2X_2R$ also resemble that of GT1 cells cotransfected with equal amounts of transcripts for $P2X_{2a}R$ and $P2X_{2b}R$. Thus, it is reasonable to speculate that the control of ATP-induced $[Ca^{2+}]_i$ signals in gonadotrophs and somatotrophs is achieved by expressing $P2X_{2a}R$ + $P2X_{2b}R$ heteromers.

In contrast to $P2X_3R$ and $P2X_2R$, the native pituitary $P2X_7R$ and its recombinant channels expressed in GT1 cells do not exhibit an obvious desensitization. In the majority of cell expressing $P2X_7R$ channels, ATP initially induces opening of a channel selective for cations, including Ca^{2+} (25). During the prolonged agonist stimulation, $P2X_7R$ opens large pores allowing permeation of larger molecules and permeabilization of cells (22, 28). This activation step is dependent on the presence of C-terminus of the receptor (22) and on the environmental temperature (29). The physiological significance of this action is still unknown. Because the increased permeability results in larger ion fluxes and leakage of small metabolites, it may cause cell swelling and vacuolization, leading to cell death by necrosis and/or apoptosis (reviewed in Ref. 25). However, whether and to what extent $P2X_7R$ is used in the control of cell death in lactotrophs is still premature to discuss.

In conclusion, the results of these investigations indicate that the specificity found in the Ca^{2+}-signaling responses induced by a common agonist is achieved by the capacity of individual receptor subtypes to generate different amplitude and kinetic patterns of Ca^{2+} responses and by the selective expression of receptors within the pituitary cell subpopulations. The ATP signaling pathway in somatotrophs and gonadotrophs is relatively simple. The majority of these cells express $P2X_{2a}R$ and $P2X_{2b}R$, and their coexpression provides an effective system for a rapid elevation in $[Ca^{2+}]_i$, which is followed by a sustained plateau Ca^{2+} response of a much smaller amplitude. We were unable to observe any other P2X receptor subtypes expressed in these cells. The most striking finding shown here is the complexity of purinergic signaling system of lactotrophs. These cells express three types of channels, $P2X_3R$, $P2X_4R$, and $P2X_7R$, as well as the Ca^{2+}-mobilizing $P2Y_2R$. The identification of P2XRs expressed in thyrotrophs and corticotrophs requires further studies. Pituitary P2X receptors can provide a wide range of Ca^{2+} signaling patterns when expressed individually or in combination. In this regard, the pituitary cells may provide a model to study how multiple receptors when expressed in excitable cells may generate different signaling in response to the same endogenous ligand.

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